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# AN ESTIMATION OF THE LIGHT-INDUCED ELECTROCHEMICAL POTENTIAL DIFFERENCE OF PROTONS ACROSS THE MEMBRANE OF *HALOBACTERIUM HALOBIUM*

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## SUMMARY

The light-dependent uptake of triphenylmethylphosphonium ($TPMP^+$) and of 5,5-dimethyloxazolidine-2,4-dione (DMO) by starved purple cells of *Halobacterium halobium* was investigated. DMO uptake was used to calculate the pH difference ($\Delta$pH) across the membrane, and $TPMP^+$ was used as an index of the electrical potential difference, $\Delta\psi$.

Under most conditions, both in the light and in the dark, the cells are more alkaline than the medium. In the light at pH 6.6, $\Delta$pH amounts to 0.6–0.8 pH unit.** Its value can be increased to 1.5–2.0 by either incubating the cells with $TPMP^+$ ($10^{-3}$ M) or at low external pH (5.5). $-\Delta$pH can be lowered by uncoupler or by nigericin. The $TPMP^+$ uptake by the cells indicates a large $\Delta\psi$ across the membrane, negative inside. It was estimated that in the light, at pH 6.6, $\Delta\psi$ might reach a value of about 100 mV and that consequently the electrical equivalent of the proton electrochemical potential difference, $\Delta\tilde{\mu}_{H^+}/F$, amounts under these conditions to about 140 mV.

The effects of different ionophores on the light-driven proton extrusion by the cells were in agreement with the effects of these compounds on $-\Delta$pH.

## INTRODUCTION

Extremely halophilic bacteria like *Halobacterium halobium* require for growth a medium containing several molar NaCl, 50–100 mM $Mg^{2+}$, and 30 mM $K^+$.

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** For any quantity $x$, $\Delta x = x_{out} - x_{in}$.

Abbreviations: CCCP, carbonylcyanide *m*-chlorophenylhydrazone; DCCD, *N,N'*-dicyclohexylcarbodiimide; $DDA^+$, diphenyldimethylammonium; DMO, 5,5-dimethyloxazoline-2,4-dione; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid; NQNO, 2-*N*-nonyl-4-hydroxyquinoline-*N*-oxide; PIPES, piperazine-*N,N'*-bis-(2-ethanesulfonic acid); SF 6847, 3,5-di-*tert*-butyl-4-hydroxybenzylidene malononitrile; TPMP, triphenylmethylphosphonium bromide.

The cells concentrate potassium up to 3–5 M internally [1–3]. Such bacteria are able to survive under conditions of minimal oxygen tension and strong illumination by forming in their membranes a purple protein, bacteriorhodopsin [4–7]. This protein functions as a light-driven proton pump [8], converting light energy into an electrochemical potential difference of protons ($\Delta\tilde{\mu}_{H^+}$) across the membrane of the cells. This potential difference can drive ATP synthesis [9–12] as suggested by Mitchell's [13, 14] chemiosmotic hypothesis.

Since both the permeability properties of the membrane and the proton translocating capacity of the bacteriorhodopsin may determine the magnitude of $\Delta\tilde{\mu}_{H^+}$, it was of interest to determine its value both in the dark and in the light. An exact measurement of the light-driven $\Delta\tilde{\mu}_{H^+}$ was not possible, due to uncertainties about the value of the electrical potential difference ($\Delta\psi$) across the cell membrane. However, in the light, the approximate values of $\Delta\tilde{\mu}_{H^+}$ and its components are similar to that of intact mitochondria [15] and intact bacteria [16–20].

## MATERIALS AND METHODS

*Cells.* Strain $R_1$ of *H. halobium* [5] was grown on a synthetic medium [21]. Bacteriorhodopsin was induced by stopping the shaking of the cellular suspension and transferring it to the light, as was described previously [12]. Cells were harvested by centrifugation and washed twice with a basal salt solution containing 4 M NaCl, 80 mM $MgSO_4$, 30 mM KCl, 0.3 mM phosphate, and 0.1 mM $CaCl_2$; final pH 6.8. Washed cells were starved overnight by stirring in basal salt containing 5 μg DNAase (Sigma)/100 mg cellular protein to reduce the high viscosity of the medium due to release of DNA from broken cells. An additional centrifugation and resuspension step in basal salt at 5–10 mg of cellular protein/ml gave preparations of intact cells that were not viscous and did not show lysis within a period of 8 h, as deduced from the fact that during this period the supernatant after centrifugation remained colourless. The bacteriorhodopsin content of the cells varied between 2 and 4 nmol bacteriorhodopsin/mg cell protein, as determined by the method of Danon and Stoeckenius [10] assuming a molar extinction coefficient for bacteriorhodopsin at 570 nm of 63 000 ($M^{-1} \cdot cm^{-1}$) [22]. Protein was determined according to the method of Lowry et al. [23], with bovine albumin as standard.

*Sources of chemicals and isotopes.* Carbonylcyanide *m*-chlorophenylhydrazone (CCCP), valinomycin, 2-(*N*-morpholino)ethanesulfonic acid (MES), piperazine-*N*,*N*′-bis-(2-ethanesulfonic acid) (PIPES), and *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) were obtained from Sigma Chemical Co.; 2-*N*-nonyl-4-hydroxyquinoline-*N*-oxide (NQNO) from General Biochemicals; *N*,*N*′-dicyclohexylcarbodiimide (DCCD) from Fluka; 5,5-dimethyloxazoline-2,4-dione (DMO) from Mann Research Laboratories; triphenylmethylphosphonium bromide (TPMP) from K and K Laboratories; nigericin from Eli Lilly and Co. and 3,5-di-*tert*-butyl-4-hydroxybenzylidene malononitrile (SF 6847) as a gift from Dr. Y. Nishizawa, Sumimoto Chemical Industry, Osaka (Japan). $^3$H-labelled TPMP (114 Ci/mol) was a gift of Dr. R. Kaback, Roche Institute of Molecular Biology, Nutley, N. J. (U.S.A.). $^{14}$C-labelled DMO (8.8 mCi/mol) and [$^{14}$C]dextran (mol. wt. 16 000) were obtained from New England Nuclear, and [$^{14}$C]sucrose, [$^{14}$C]sorbitol, and $^3H_2O$ from Amersham Radiochemical Centre.

*Tracer distribution between the bacteria and the medium.* The microcentrifugation technique, as described previously for chloroplasts [24], chromatophores [25], and mitochondria [15], was used to determine the distribution of tracer molecules between the cells and the medium. Unless otherwise stated, cells were incubated at 5–10 mg protein/ml of basal salt at 25 °C for 25 min in the presence of the proper tracers, 30 $\mu$M TPMP, and 50 mM PIPES/NaOH buffer; final pH 6.6. In general three parallel experiments were carried out, one to determine internal water space of the cell pellet with $^3H_2O$ at 10 $\mu$Ci/ml and the $^{14}$C-labelled sugar compound at 1–2 $\mu$Ci/ml, one to determine $\Delta$pH with $^3H_2O$ and $^{14}$C-labelled DMO at the same specific activity, respectively, and one to determine the uptake of $TPMP^+$ by the cells with $^3$H-labelled TPMP at 0.3 $\mu$Ci/ml. Aliquots (200 $\mu$l) of such bacterial suspensions were added in triplicate to transparent 400-$\mu$l microcentrifuge tubes and put into a Beckman 152 microfuge. Light samples were illuminated in the microfuge for 3 min by means of two 250 W reflector lamps shining through the transparent deck cover. A 3 cm water filter was placed in between the lamps and the deck of the microfuge. The samples were then centrifuged under illumination for 5 min. The light intensity within the microfuge during preillumination and centrifugation was $3 \cdot 10^5$ ergs $\cdot$ cm$^{-2} \cdot$ s$^{-1}$. Dark controls were kept in the dark in the centrifuge for 3 min and were centrifuged in the dark for 5 min. Both in the light and in the dark the temperature in the centrifuge rose during the centrifugation to 30–35 °C. The time needed to spin down half of the cells from the supernatant was estimated to be 30 s. From the supernatant fraction a 50 $\mu$l sample was mixed with 0.5 ml 1 M NaOH. A slice from the bottom of the pellet was cut and added to 0.3 ml 1 M NaOH. The NaOH fractions were slowly shaken overnight at 35 °C in stoppered tubes to dissolve the protein. 100-$\mu$l samples of the NaOH solutions were added to 8.5 ml liquid scintillation fluid, prepared according to Bray [26], and were counted in a Tricarb scintillation counter (Packard M 3900). The $^{14}$C and $^3$H activities of the different samples were corrected for spill-over and quenching using a channel-ratio method. Small samples were taken from each of the NaOH solutions of the pellet fractions to determine the amount of protein cut from the pellets, using the method of Lowry et al. [23], with bovine albumin as a standard.

*The water space of the cells and the total water content of the pellet.* The total water content of the cell pellet was calculated from the ratio of activities of $^3H_2O$ in the pellet and in the supernatant, and expressed in $\mu$l/mg protein. The internal water space $\alpha$ was determined as a percentage of the total water content of the pellet by using $^3H_2O$ and [$^{14}$C] sucrose, [$^{14}$C] sorbitol, or [$^{14}$C] dextran, and is given by:

$$\alpha = \left[1 - \frac{(^{14}C/^{3}H)_{pellet}}{(^{14}C/^{3}H)_{supernatant}}\right] \times 100\,\% \qquad (1)$$

*The determination of $\Delta$pH across the cellular membrane.* Intact *H. halobium* cells extrude protons upon illumination [8]. The weak acid $^{14}$C-labelled DMO, used to estimate $\Delta$pH [27, 28, 15], is accumulated in intact bacteria relative to the medium. For DMO the following relation holds at equilibrium [30]:

$$\frac{A^T_{in}}{A^T_{out}} = \frac{1/K_a + 1/H^+_{in}}{1/K_a + 1/H^+_{out}} \qquad (2)$$

where $A^T_{in}$ and $A^T_{out}$ are the total DMO concentrations in the cells and in the medium, respectively, and $K_a$ is the dissociation constant of DMO. The p$K_a$ value of DMO was

found to be 6.2 at 22 °C, both at low and at high (4 M NaCl) ionic strength. The pH electrode used for this determination was calibrated at low ionic strength. Similarly, all pH measurements of cellular suspensions in basal salt solutions were carried out with pH electrodes calibrated at low ionic strength. For the calculation of $\Delta$pH according to Eqn. 2, we assumed a p$K_a$ value for DMO of 6.30 at 30 °C [28]. The apparent accumulation factor for DMO in the cells equals the ratio ($^{14}$C-labelled DMO/$^3H_2O$)$_{pellet}$/($^{14}$C-labelled DMO/$^3H_2O$)$_{supernatant}$. From this factor, correcting for the external water space of the pellet, and using the p$K_a$ value for DMO, $\Delta$pH across the cell membrane was calculated [28].

*The determination of the TPMP$^+$ uptake by the cells.* The accumulation of TPMP$^+$ by the cells is given as the TPMP$^+$ uptake ratio. It equals the ratio ($^3$H-labelled TPMP$^+$/mg protein)$_{pellet}$/($^3$H-labelled TPMP$^+$/$\mu$l)$_{supernatant}$. This ratio is proportional to the ratio [TPMP$^+$]$_{bound}$/[TPMP$^+$]$_{free}$, if all the TPMP$^+$ taken up by the cells is considered bound. Alternatively, if all the TPMP$^+$ taken up is considered free in the internal water space of the cells, the uptake ratio is proportional to the ratio [TPMP$^+$]$_{in}$/[TPMP$^+$]$_{out}$. In the discussion we try to relate the TPMP$^+$ uptake ratio to the electrical potential difference $\Delta\psi$ across the membrane, and to estimate $\Delta\tilde{\mu}_{H^+}$ from $-\Delta$pH and $\Delta\psi$.

*pH measurements.* Changes in pH in the medium of the cells were measured with a Radiometer GK 2321C combined glass electrode connected to a Radiometer PHM 64 pH meter. The thermostat vessel was maintained at 30 °C, and contained 3 ml of a medium, consisting of basal salt, cells at 0.5–2.0 mg protein/ml, and 5 mM PIPES/NaOH buffer, final pH 6.6. Illumination was provided by means of a 500 W slide projector at a light intensity of $10^6$ ergs $\cdot$ cm$^{-2}$ $\cdot$ s$^{-1}$.

## RESULTS

### *The internal water fraction of the total water space of the cell pellet*

Fig. 1 shows the intracellular fraction of the total water content of the cell pellet. With dextran, sucrose, or sorbitol, values between 50 and 60 % are found, regardless of whether non-purple cells (Fig. 1B) or purple cells (Fig. 1A) were used and whether the latter were illuminated or not. The values found with dextran were always somewhat higher than those found with sucrose or sorbitol (Fig. 1A). This might indicate that dextran cannot permeate the cell wall [4], but that sucrose and sorbitol can and are only stopped by the cell membrane [4]. From the time dependence seen in Fig. 1, it can be concluded that dextran and sucrose hardly penetrate into the internal water space of the cells and that sorbitol does so only slowly.

Under a large variety of conditions values between 4.0 and 5.5 $\mu$l/mg protein were found for the total water content of the pellet (not shown). For the calculations of $\Delta$pH, $\Delta\psi$, and $\Delta\mu_{H^+}$ (see Discussion), it was assumed that the external water space of the cell pellet equals its internal water space, and has a value of 2.5 $\mu$l/mg protein, i.e. half that of the total water content of the pellet.

### *Light-dependent $\Delta$pH and TPMP$^+$ uptake by the cells*

We tested the light-dependent uptake of three lipid-soluble cations by *H. halobium* cells. These cations, $^{86}Rb^+$, $^{42}K^+$ (plus valinomycin), and TPMP$^+$, reportedly distribute according to $\Delta\psi$ across mitochondrial [31–33, 15] and bacterial

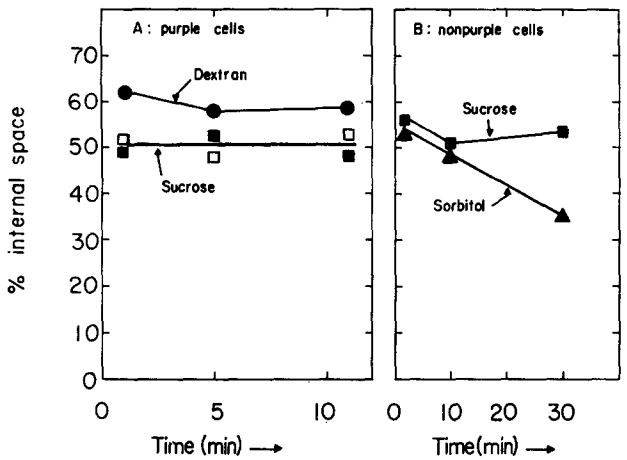


Fig. 1. The internal water space as a percentage of the total water space of the pellet. Cells were incubated as described under Materials and Methods, except that at $t = 0$ [$^{14}$C]dextran, [$^{14}$C]-sucrose, or [$^{14}$C]sorbitol were added to the cellular suspension preincubated with $^3H_2O$. At the time indicated in the figure the cells were spun down. Open symbols: light values; closed symbols: dark values.

[17, 20, 29, 34] membranes. The former two cations, even in the presence of $10^{-5}$ M valinomycin, distribute orders of magnitude too slowly to monitor $\Delta\psi$ across the membrane of *H. halobium* in a satisfactory way. However, $\Delta\psi$ is the driving force for the slow uptake observed for these ions (Garty, H. and Caplan, S. R., unpublished). Schuldiner and Kaback [29] have introduced the use of $^3$H-labelled TPMP$^+$ distribution as a method of $\Delta\psi$ estimation in *Escherichia coli* membrane vesicles. With *H. halobium* cells, after the light is switched on at $t = 0$, the rates of redistribution of DMO and TPMP$^+$ across the cell membrane are fast (Fig. 2). However, the difference observed between the light and the dark values of the TPMP$^+$ uptake ratio are rather small. Since the high dark values are probably caused by binding to the membrane of part of the TPMP$^+$ taken up by the cells (see below), we prefer to present our data as the TPMP$^+$ uptake ratio, rather than to give the ratio $[TPMP^+]_{in}/[TPMP^+]_{out}$ or to convert this ratio directly to $\Delta\psi$.

In Table I data are given on $-\Delta$pH and the TPMP$^+$ uptake ratio of the cells, both in the light and in the dark. Inhibitors of respiration, which effect the rate of oxygen uptake by the cellular suspensions (not shown), show different effects on $\Delta$pH and TPMP$^+$ uptake. NQNO and rotenone hardly exert any influence, but 10 mM $NaN_3$ reduces the dark value of $-\Delta$pH and has a small influence on both the light and dark values of the TPMP$^+$ uptake. However, this inhibitor also shows effects on the light-driven proton extrusion by the cells typical of an uncoupler (see below). Therefore, we conclude that its small effects on $\Delta$pH and on the TPMP$^+$ uptake ratio (Table I) are due to weak uncoupling rather than to inhibition of residual respiration still present in the starved cells. Since neither the other inhibitors tested (Table I) nor anaerobiosis (not shown) are effective in reducing the high dark values of $-\Delta$pH and of the TPMP$^+$ uptake ratio, it is concluded that these values are not caused by residual respiration.

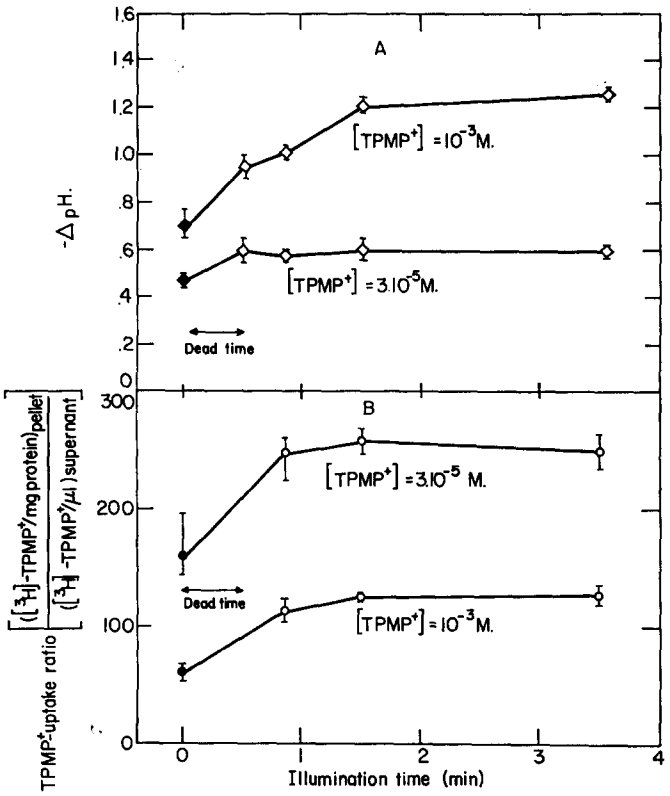


Fig. 2. Kinetics of light-induced $\Delta$pH formation (A) and of changes in the $TPMP^+$ uptake ratio (B). Cells were incubated with $^3H_2O$ and with $^{14}C$-labelled DMO or with $^3H$-labelled TPMP, as described under Materials and Methods, except that either $3 \cdot 10^{-5}$ or $10^{-3}$ M $TPMP^+$ was present and illumination of the samples in the microcentrifuge was started at $t = 0$. The points given at this time represent dark controls (closed symbols). The time given on the abscissa is the sum of the dead time of the centrifugation system (30 s, see Materials and Methods) and that which elapsed between the moments of switching on the light and switching on the centrifuge (open symbols). Each symbol gives the position of the average of triplicate values; each bar represents the deviation of the extreme values.

The energy transfer inhibitor DCCD increases the values of the $TPMP^+$ uptake ratio of the cells, especially in the light (Table I), probably since it blocks leaks through the ATPase pathway. (Compare with chloroplasts, where phosphorylating conditions decrease and Dio-9 increases the value of $\Delta$pH across the thylakoidal membrane [35].)

The kinetics and final extents of $\Delta$pH formation and $TPMP^+$ uptake ratio after switching on the light at $t = 0$, are shown in Fig. 2. At a relatively low concentration of $TPMP^+$ (30 $\mu$M), the times needed to achieve steady state $\Delta$pH and $TPMP^+$ uptake are shorter than the "dead" time of the centrifugation system (Fig. 2). At a $TPMP^+$ concentration of 1 mM the formation of $\Delta$pH can be followed kinetically (Fig. 2A). At this level of $TPMP^+$ the light-induced pH changes observed in the cellular medium also become bigger in extent and slower in formation (see below, Fig. 6). Therefore one can conclude that under such conditions it is the formation of

TABLE I

INFLUENCE OF METABOLIC INHIBITORS ON THE LIGHT AND DARK VALUES OF $-\Delta$pH AND THE $TPMP^+$ UPTAKE RATIO OF INTACT *H. HALOBIUM* CELLS

Experimental conditions as described in Materials and Methods. The cells were preincubated with the respective inhibitors for 10 min, except for DCCD, where a preincubation time of 60 min was used before the cells were transferred into the microfuge tubes. The values given are the averages of triplicate experiments. The deviations are those between the most extreme and the average value.

| Experiment | Addition | $-\Delta$pH | | $TPMP^+$ uptake ratio | |
|---|---|---|---|---|---|
| | | Dark | Light | Dark | Light |
| 1 | None | 0.45±0.03 | 0.61±0.03 | 230±25 | 340±6 |
| | $NaN_3$ (10 mM) | 0.24±0.03 | 0.60±0.04 | 150±30 | 225±23 |
| | NQNO (5 μM) | 0.45±0.02 | 0.63±0.03 | 220±20 | 265±20 |
| | DCCD (10 μM) | 0.44±0.02 | 0.61±0.04 | 378±20 | 838±20 |
| | $NaN_3$ (10 mM) DCCD (10 μM) | 0.33±0.02 | 0.56±0.03 | 150±20 | 320±25 |
| | NQNO (5 μM) DCCD (10 μM) | 0.41±0.04 | 0.63±0.03 | 310±40 | 750±120 |
| 2 | None | 0.58±0.04 | 0.79±0.01 | 160±9 | 240±20 |
| | NQNO (2 μM) | 0.54±0.01 | 0.81±0.02 | 170±10 | 229±20 |
| | NQNO (2 μM) rotenone (4 μg/ml) | 0.56±0.02 | 0.75±0.03 | 135±10 | 150±10 |

$\Delta$pH rather than the redistribution of DMO that is rate limiting. After switching off the light, $-\Delta$pH drops to its original dark level (not shown). Increasing concentrations of $TPMP^+$ steadily decrease the values of the $TPMP^+$ uptake ratio (Fig. 3), and even at relatively high $TPMP^+$ no constant uptake level is reached, both in the light and in the dark. Above a concentration of $10^{-4}$ M, $TPMP^+$ increases $-\Delta$pH (Figs. 2A and 3A). The other experiments reported in this section were all carried out at $TPMP^+$ concentrations of $3 \cdot 10^{-5}$–$1 \cdot 10^{-4}$ M, i.e. just below the concentration at which this compound starts to influence $\Delta$pH.

The influence of two ionophores on $-\Delta$pH and the $TPMP^+$ uptake ratio is shown in Fig. 4. The uncoupler of oxidative phosphorylation CCCP [36] decreases the light and the dark values of both $-\Delta$pH and of the $TPMP^+$ uptake ratio, but of the two, more uncoupler is required to produce an appreciable effect on $-\Delta$pH (Fig. 4A). At high concentrations of CCCP the $TPMP^+$ uptake ratio reaches the same minimal level for light and dark. The same minimal level was also found with another uncoupler, SF 6847 [37], which was about as effective as CCCP on *H. halobium* cells (not shown). However, both uncouplers are much less active on these cells than they are on rat liver mitochondria [37–39].

Valinomycin reduces the value of the $TPMP^+$ uptake ratio somewhat, but hardly effects $-\Delta$pH (Fig. 4B). Nigericin has the opposite effect: it diminishes $-\Delta$pH, especially in the dark, but it only decreases the ratio of the $TPMP^+$ uptake slightly (not shown).

Fig. 5 shows how the pH of the external medium effects the $TPMP^+$ uptake ratio and $-\Delta$pH. The two parameters show opposite behaviour: at low external pH,

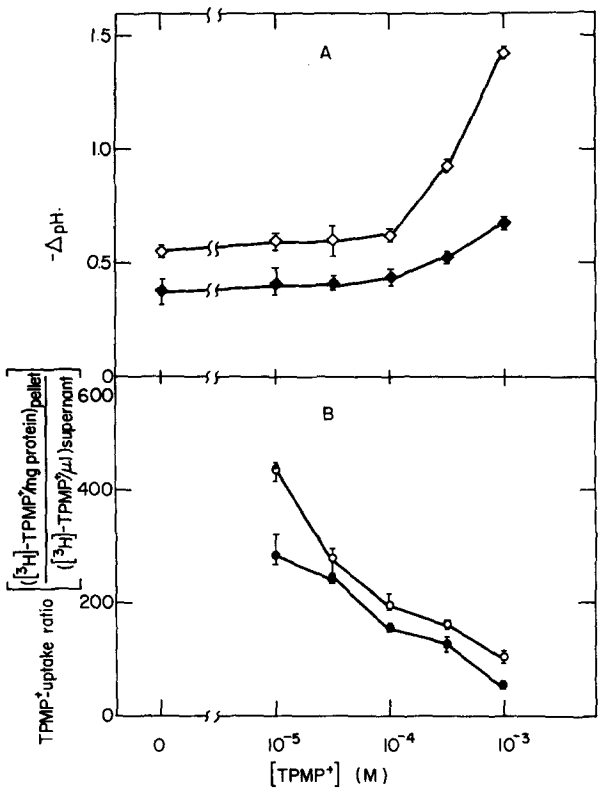


Fig. 3. The influence of [TPMP$^+$] on the $\Delta$pH (A) and the TPMP$^+$ uptake ratio (B). Cells were incubated as described under Materials and Methods, except that concentrations of TPMP$^+$ were present as indicated on the abscissa. Open symbols, light values; closed symbols, dark values (see legend to Fig. 2).

the value of $-\Delta$pH is relatively large and that of the TPMP$^+$ uptake ratio is low, while the reverse is true at high external pH. Between external pH values of 5.5 and 7.0 the internal pH is essentially constant, and only rises when the external pH is above the latter value (Fig. 5, inset).

*pH changes observed in the medium of the cells*

Below we compare some of the reagents affecting the light-induced pH changes in the cell suspension with their effects on $\Delta$pH. The light-induced proton extrusion by intact cells of *H. halobium* shows a complex behaviour [8, 10–12]. At high light intensity ($10^6$ erg $\cdot$ cm$^{-2}$ $\cdot$ s$^{-1}$) an initial alkalinization of the medium is followed by a net acidification. After switching off the light, a further acidification is followed by a net alkalinization [8, 12, 40]. At lower light intensities than $10^5$ ergs $\cdot$ cm$^{-2}$ $\cdot$ s$^{-1}$ only a net light-induced alkalinization and a net dark-induced acidification are observed [12, 40]. At an intermediate light intensity of $3 \cdot 10^5$ ergs $\cdot$ cm$^{-2}$ $\cdot$ s$^{-1}$ we could only detect uptake of TPMP$^+$ and DMO (see above). Therefore, under those conditions, the proton pump of *H. halobium* must be outwardly directed and the

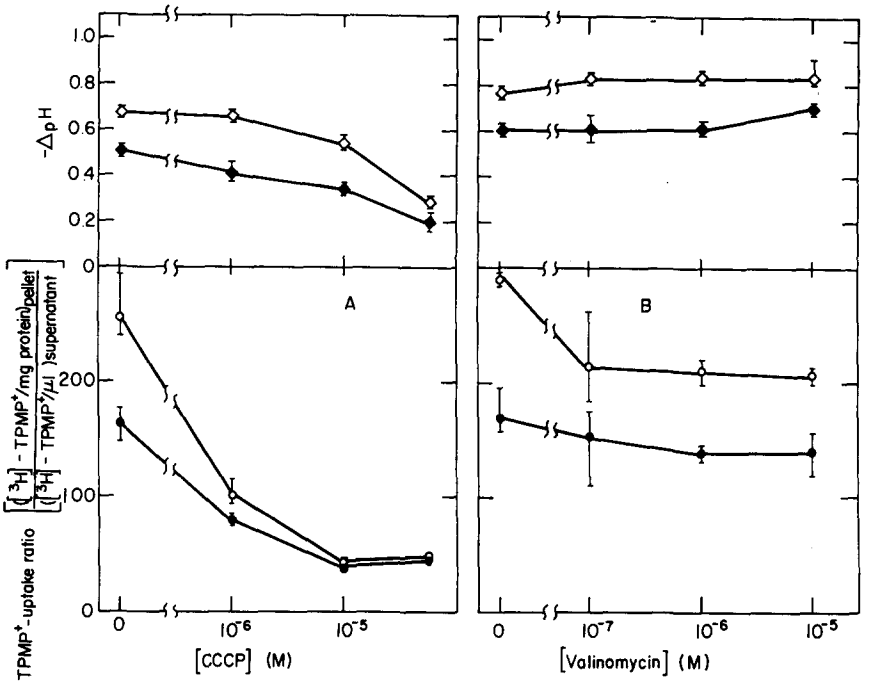


Fig. 4. The influence of CCCP (A) and valinomycin (B) on the $\Delta$pH and the $TPMP^+$ uptake ratio. Cells were treated as described under Materials and Methods, except that in A $10^{-4}$ M $TPMP^+$ was present, and that the cells were preincubated for 10 min with the ionophores at concentrations indicated. Open symbols, light values; closed symbols, dark values (see legend to Fig. 2).

initial alkalinization and acidification effects cannot be the direct result of proton pumping, but may be caused by ATP synthesis [11, 12] or other energy-requiring processes. To minimize the initial effects, all our experiments on light-induced proton movements were carried out at saturating light intensity.

Addition of $10^{-4}$ M TPMP or more to the cells induces a release of protons from the cells in the dark. In the light, the extent of net proton extrusion is increased (Fig. 6). Both effects can be explained as due to a $H^+$-$TPMP^+$ exchange that increases $-\Delta$pH both in the dark and in the light (Fig. 3).

The uncouplers CCCP and SF 6847 are almost equally effective in abolishing the net light-induced proton extrusion by the cells (Fig. 7). In the light the initial alkalinization is more sensitive to uncoupler than the final acidification. In the dark the initial acidification of the medium is enhanced by low concentrations of uncoupler, but abolished by higher concentrations, as is the final alkalinization. The respiratory inhibitor $NaN_3$ at 10 mM causes effects similar to those of $10^{-6}$ M CCCP on the pH changes in the medium. Therefore it is concluded that $NaN_3$ also acts as a weak uncoupler on the system.

Nigericin, when added in the dark to the cells, induces an uptake of protons. In the light, approximately the same amount of protons are released as were taken up in the dark (Fig. 8). The uptake of protons in the dark is most probably due to a $K^+$-$H^+$ exchange process, driven by the high internal potassium concentration [1–3].

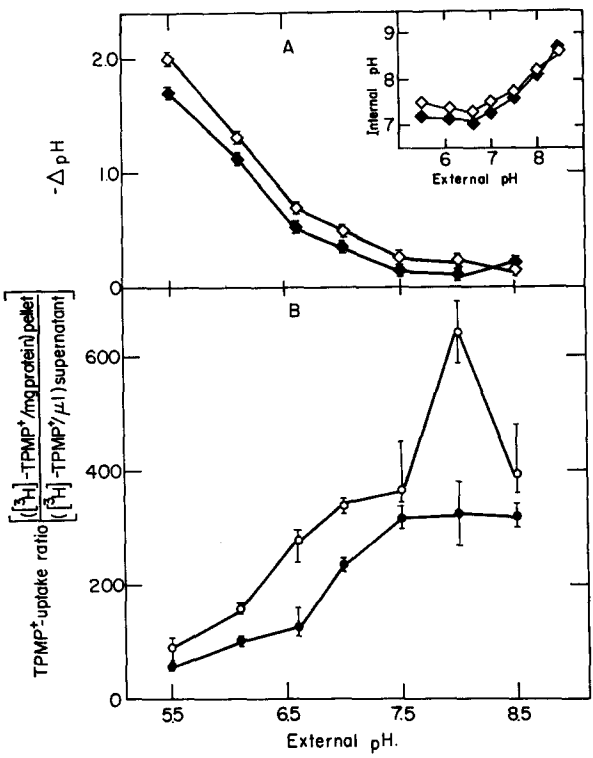


Fig. 5. The influence of the external pH on $\Delta$pH (A) and the $TPMP^+$ uptake ratio (B). Cells were incubated as described under Materials and Methods, except that instead of PIPES/NaOH buffer, pH 6.6, either 50 mM MES/NaOH buffer (final pH 5.5 and 6.1), 50 mM PIPES/NaOH buffer (final pH 6.6 and 7.1), or 50 mM HEPES/NaOH buffer (final pH 7.5, 8.0, and 8.5) was present. Open symbols, light values; closed symbols, dark values (see legend to Fig. 2).

This reduces the dark $-\Delta$pH, increases the internal proton content, and therefore allows the light-driven proton pump to transport more protons from the cell before substantially decreasing $-\Delta$pH.

Valinomycin, even at $3 \cdot 10^{-5}$ M, does not influence pH changes in the medium due to the light-driven proton pump (not shown), in contrast to the small influence that it exerts on the $TPMP^+$ uptake ratio (Fig. 4B).

## DISCUSSION

Purple *H. halobium* cells show light-induced uptake of DMO and of $TPMP^+$. From the former the pH difference ($-\Delta$pH) across the cell membrane was calculated. Under most conditions, both the light and the dark values of $-\Delta$pH are small (below one pH unit), and only increase at low external medium pH (Fig. 5) or at high $TPMP^+$ concentrations (Fig. 3). The small $\Delta$pH values observed for *H. halobium* are similar to those of other energy-converting systems like intact mitochondria [15, 28] or intact bacteria [18, 19] where the membrane orientation is the same.

In a number of studies it has been shown that the uptake by bacteria or sub-

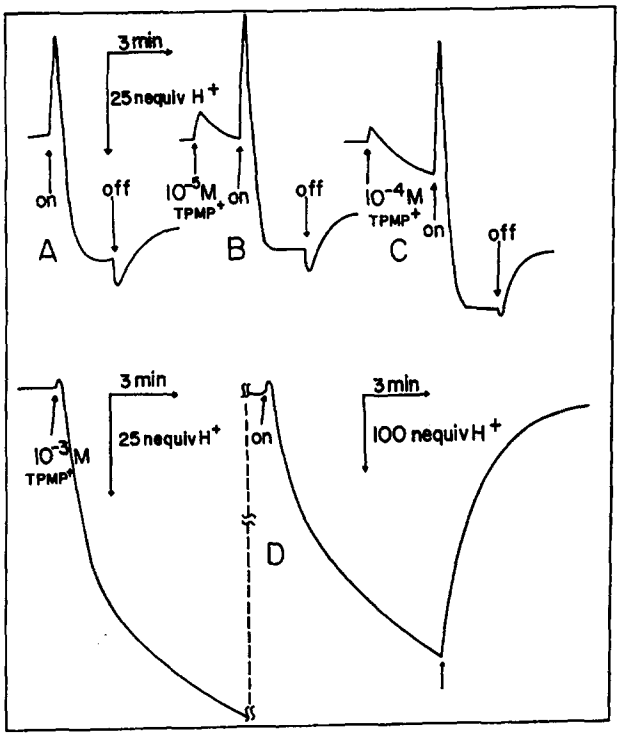


Fig. 6. The influence of $TPMP^+$ on the proton pump. Details of the incubation mixture are described under Materials and Methods, except that the cellular protein concentration was 0.8 mg/ml. At the left-hand arrow of each trace $TPMP^+$ was added in the dark to the suspension at the concentration indicated. Cells were illuminated (arrow "on"). Later on the light was switched off (arrow "off"). Note the difference in sensitivity of the scale at $10^{-3}$ M $TPMP^+$.

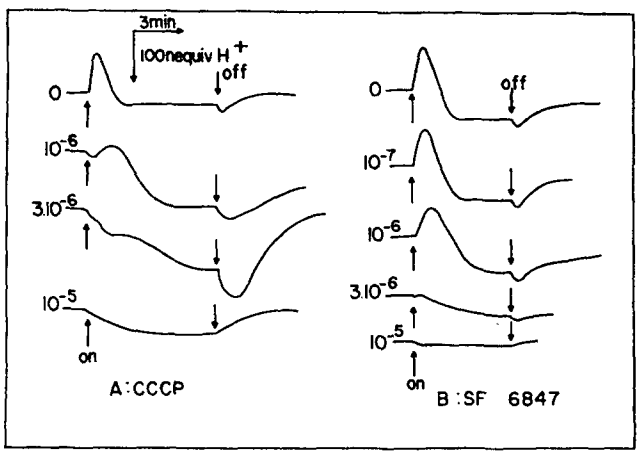


Fig. 7. The influence of uncouplers on the proton pump. Cells at 1.1 mg/ml suspension medium (see Materials and Methods) were incubated with the concentration of uncouplers as indicated in the figure. At "on" and "off", the light was switched on and off, respectively.

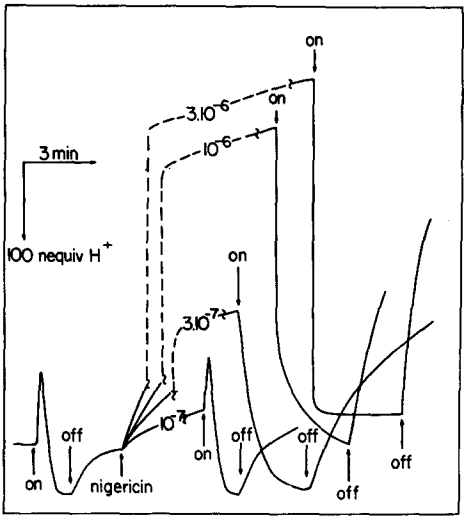


Fig. 8. The influence of nigericin on the proton uptake. Cells at 1.7 mg/ml were exposed to one light-dark cycle (left-hand part of the figure). At the arrow nigericin was added in the dark to the suspension at the concentrations indicated in the respective traces. At "on" and "off", the light was switched on and off, respectively.

bacterial particles of lipid-soluble cations like $TPMP^+$ or dibenzyldimethyl ammonium ($DDA^+$) in the presence of tetraphenylboron is driven by the electrical potential difference ($\Delta\psi$) across the membrane [16, 17, 20, 31, 34]. With *E. coli* membrane vesicles it was recently shown that the values of the uptake ratio of $TPMP^+$, $DDA^+$ (in the presence of tetraphenylboron) and of $Rb^+$ (in the presence of valinomycin) are almost identical and that therefore $\Delta\psi$ can be calculated from this ratio [29]. The following characteristics of the $TPMP^+$ uptake by *H. halobium* cells are compatible with a distribution of $TPMP^+$ across the membrane according to $\Delta\psi$. (1) In the light a fast uptake of $TPMP^+$ by the cells is observed (Table I and Fig. 2). This indicates that the electrical potential difference across the membrane is negative inside the cell as expected, i.e. generated by an outwardly directed proton pump. (2) Uncouplers of oxidative phosphorylation drastically diminish the value of the $TPMP^+$ uptake ratio, both in the light and in the dark (Fig. 4A). It is to be expected that $\Delta\psi$ would be uncoupler sensitive, since it is generated by a proton pump (cf. Fig. 7). (3) Under conditions where $-\Delta$pH across the membrane is high and one expects a low value for $\Delta\psi$ (i.e. at low external pH or at high $TPMP^+$ concentrations), the $TPMP^+$ uptake ratio is comparatively low (Figs. 5, and 2 and 3, respectively).

However, possible binding of $TPMP^+$ to the cells represents a serious problem for exact $\Delta\psi$ calculations from the $TPMP^+$ uptake ratio. The extremely high values found for this ratio in the dark are not due to residual respiration (Table I). It cannot be a distribution of $TPMP^+$ according to a Donnan potential across the membrane, since it is expected that in the dark protons would also accumulate, which was not observed. These high values might, however, be due entirely or in part to binding of $TPMP^+$. Sub-bacterial particles prepared from *H. halobium* with an "inside-in" membrane orientation [41] and isolated purple membranes have, in the dark, a very

high uncoupler-insensitive $TPMP^+$ uptake ratio, indicating binding (Garty, H. and Eisenbach, M., unpublished). In rat liver mitochondria the $TPMP^+$ uptake ratio is also always larger than that of $Rb^+$ in the presence of valinomycin, particularly in the presence of uncoupler (Rottenberg, H., unpublished).

To correct the $TPMP^+$ uptake ratio for binding of $TPMP^+$ in order to calculate the free concentration of $TPMP^+$ inside the cell is not simple. In the case of *H. halobium*, unlike in other systems [29, 15], no comparison can be made with the distribution of $Rb^+$ across the membrane (see above). If one nevertheless calculates $\Delta\psi$ according to Eqns. 3 and 4:

$$\Delta\tilde{\mu}_{TPMP^+} = RT \ln \frac{[TPMP^+]_{out}}{[TPMP^+]_{in}} + zF\,\Delta\psi \tag{3}$$

and at equilibrium ($\Delta\tilde{\mu}_{TPMP^+} = 0$)

$$\Delta\psi = (2.3\,RT/F) \log \frac{[TPMP^+]_{in}}{[TPMP^+]_{out}} \tag{4}$$

Assuming as a first approximation that no binding of $TPMP^+$ occurs, one obtains the values for $\Delta\psi$ given in Figs. 9 and 10, where the experiments of Figs. 4a and 5, respectively, are replotted. Assuming as a second approximation that the constant level of the $TPMP^+$ uptake ratio obtained at high uncoupler concentration (Fig. 4A) represents the amount of $TPMP^+$ bound to the cells, and also that this binding is potential independent, one obtains corrected values for $\Delta\psi$ of 111 mV compared to 116 mV (in the light), and 97 mV compared to 104 mV (in the dark). In fact, however, data on mitochondria indicate that the binding is potential dependent (Rottenberg,

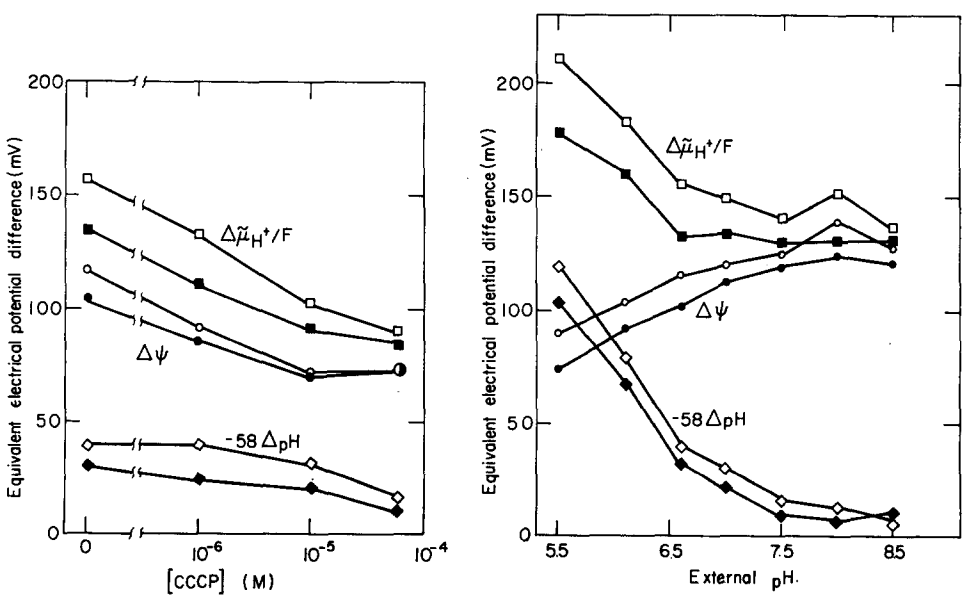


Fig. 9. The experiment of Fig. 4a, replotted on a scale of normalized electrical potential difference (see Discussion). Symbols: ◆, ◇, $-58\,\Delta pH$; ●, ○, $\Delta\psi$; ■, □, $\Delta\tilde{\mu}_{H^+}/F$. Open symbols, light values; closed symbols, dark values.

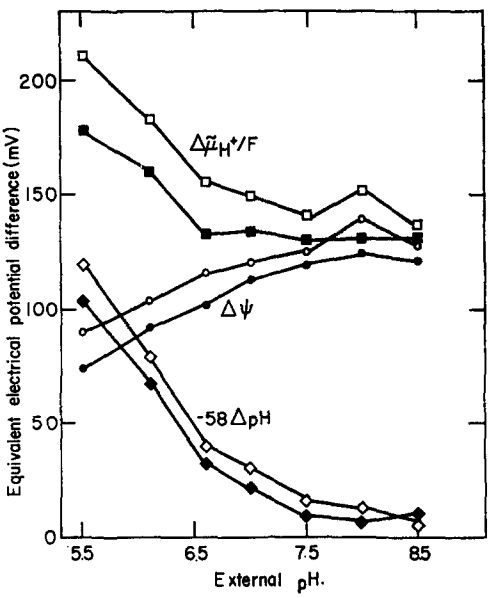


Fig. 10. The experiment of Fig. 5 replotted on a scale of normalized electrical potential difference (see Discussion). Symbols: see legend to Fig. 9.

H., unpublished). Finally, one might assume that the high dark values of the $TPMP^+$ uptake ratio are entirely due to binding and correct the light values for it. In this case the calculated potential drops from 116 to 95 mV.

It will be clear that an exact calculation of $\Delta\psi$ from the $TPMP^+$ uptake ratio is not possible. However, it is evident that at pH 6.6 at low light intensity the value of $\Delta\psi$ across the membrane is between +95 and +116 mV. Thus intact *H. halobium* cells generate an electrochemical potential difference of protons ($\Delta\tilde{\mu}_{H^+}$) across the membrane consisting of a $-\Delta$pH and a $\Delta\psi$ component (Fig. 9), where $\Delta\tilde{\mu}_{H^+}$ is given by:

$$\Delta\tilde{\mu}_{H^+} = RT \ln \frac{H^+_{out}}{H^+_{in}} + zF\,\Delta\psi \tag{5}$$

or expressed in mV [9, 10]:

$$\Delta p \equiv \Delta\tilde{\mu}_{H^+}/F = -58\Delta\text{pH} + \Delta\psi. \tag{6}$$

The value of $\Delta\tilde{\mu}_{H^+}/F$ in the light lies between 135 and 157 mV. From Fig. 10 it can be seen that at pH 5.5 the contribution of the $-\Delta$pH component to $\Delta\tilde{\mu}_{H^+}/F$ becomes the major one.

We show in this paper that *H. halobium* has a cell membrane that is rather impermeable to smaller sugar molecules and across which it is possible to build up a $\Delta\tilde{\mu}_{H^+}$ of considerable magnitude. This result is in contrast to the claim that another extremely halophilic bacterium species has a membrane that is permeable to all kinds of small molecules and ions [2, 42, 43]. This discrepancy is remarkable because the different halobacteria have similar ion contents [1–3] and membrane lipid components [44], and appear to be able to form bacteriorhodopsin (refs. 4–7, and Danon, A. and Bakker, E. P., unpublished).

Several results presented in this paper are supported by data from the literature. Thus, the pH dependence of $\Delta$pH (Fig. 4), which is very similar to that of *E. coli* [18], is also in accordance with the data of Kanner and Racker [45] on the pH dependence of the proton pump in broken cells of *H. halobium*. The concentration of $TPMP^+$, $10^{-3}$ M, which induces an increase of both $-58\Delta$pH and the extent of proton uptake (Figs. 3 and 6), sharply diminishes the rate of influx of amino acids into broken cells [40] and also of $^{86}Rb^+$ into intact cells (Garty, H. and Caplan, S. R., unpublished). Finally, the small effect of valinomycin at low light intensity on the $TPMP^+$ uptake ratio (Fig. 4b), and the absence of an effect at high light intensity on the net proton extrusion (Results), are in agreement with reportedly small effects on $K^+$ or $Rb^+$ movements (refs. 45 and 46, and Garty, H. and Caplan, S. R., unpublished) across other *Halobacterium* membranes or on their proton pump [4]. Apparently, valinomycin induces its effect on *H. halobium* cells of uncoupling of light-induced phosphorylation [11], or of stimulation of ATP synthesis when added in the dark to the cells [12], without involvement of a big net movement of $K^+$.

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